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TECHNICAL MANUSCRIPT 244

COCCIDIOIDIN SENSITIVITY IN GUINEA PIGS  
IMMUNIZED WITH KILLED ARTHROSPORES

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U.S. ARMY BIOLOGICAL LABORATORIES  
Fort Detrick, Frederick, Maryland

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Special Operations Division  
DIRECTORATE OF DEVELOPMENT

Project 1C522301A059

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In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

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### ABSTRACT

Guinea pigs injected subcutaneously with dead *C. immitis* arthrospores developed sensitivity to coccidioidin skin testing after 1½ weeks. At that time indurations 5 mm or greater in diameter were seen at 8 and 24 hours. Later indurations, most often with erythema, were observed at 3, 5, 8, 24, and 48 hours. Gross reactions were not completely specific to the immunized animals and one nonimmunized control animal developed similar indurations. Histologic features of a delayed hypersensitivity also appeared 1½ weeks after immunization. This reaction consisted first of early neutrophilic infiltration of the dermis and subcutaneous tissues uniformly present at 3 hours and less marked by 48 hours. The second cellular feature was a later, variable mononuclear cell infiltration and was usually most marked at 24 and 48 hours. The acute inflammatory reaction was produced in both sensitized and control animals. The two groups differed in the degree of mononuclear cell infiltration, which occurred sooner and was more marked in the sensitized animals than in the controls.

All agar-gel precipitin-inhibition reactions were negative on sera from all animals tested.

No immediate anaphylactic reaction was produced in immunized animals by intravascular injection of coccidioidin.

Peritoneal cellular exudate, predominantly large mononuclear cells, from immunized, coccidioidin-positive donors transferred the hypersensitivity to control animals. Sera from similar immunized coccidioidin donors failed to transfer the hypersensitivity.

## I. INTRODUCTION

This investigation was designed to characterize the skin test response in guinea pigs after they are immunized against coccidioidomycosis with whole, killed arthrospores of Coccidioides immitis. In other studies immunized animals have been challenged by various routes to assay the effectiveness of the vaccines against this disease.<sup>1</sup> With the improvement of these vaccines and their use in humans,<sup>2</sup> a method other than challenge to assay their effectiveness must be found. The coccidioidin skin test, rather than serologic determinations, is a possible method because positive results are obtained in various experimental animals during immunization.<sup>3-5</sup> Immunized animals with positive skin tests also had some degree of protection against challenge.<sup>6-8</sup> Serologic tests during the immunization period using antigens without adjuvants were uniformly negative.<sup>6-8</sup> Killed arthrospores plus incomplete Freund's adjuvant elicited in monkeys at 30 and 90 days agar gel precipitin titers that receded to zero in the majority of animals at 6 months postimmunization.<sup>9</sup>

The techniques used to characterize the skin test in this experiment are: gross observations, histologic examinations, serologic determinations, anaphylaxis, and passive transfer of skin test sensitivity by cells and sera.

## II. MATERIALS AND METHODS

### A. ANIMALS USED

Male, Hartley strain, guinea pigs weighing 250 to 300 grams, born and raised at the Fort Detrick Animal Farm, were used in the investigation. Each animal was identified by a specific number tattooed on its ear.

### B. ANTIGEN AND SENSITIZATION

The antigen was killed arthrospores of C. immitis strain Silveira. These spores were grown, killed, and checked for viability as described in another report. For injection, the cells were suspended in tri-ethanolamine oleate and saline solution. The experimental series of animals were sensitized by subcutaneous injections in the shoulder area. Three 0.5-ml injections containing a total of 24 mg of antigen (dry weight) were given.

### C. COCCIDIOIDIN AND SKIN TESTING

Coccidioidin used in this study was prepared in these laboratories according to the method of Smith et al.<sup>4</sup> In guinea pigs it produced indurations that were within one mm of the size of those from a standard Smith-lot tested in the same animals.

None of the animals was skin tested before to avoid possible sensitization by skin testing with potent coccidioidin.<sup>8</sup> Skin testings were made at 1, 1½, 2, 2½, 3, and 3½ weeks after sensitization. Skin tests were done on the abdomen and each site was marked with dye. At each time period, four separate animals were used, three sensitized and one non-sensitized animal for a control. Each of the four animals was given a total of six skin tests such that at the time of sacrifice each animal had separate 48-, 24-, 8-, 5-, 3-, and ½-hour reactions.<sup>10</sup>

### D. GROSS OBSERVATIONS

Skin tests with indurations of 5 x 5 mm or greater were considered positive responses. The development of edema and erythema was also noted.

### E. HISTOPATHOLOGY

Two of the experimental and the one control animal were sacrificed by injecting 120 mg of Nembutal intraperitoneally. After sacrifice the entire skin covering the abdomen was removed. Special care was taken to include the subcutaneous tissue to the muscle layer. The tissues were pinned on wood and fixed in 10% buffered formalin. Three pieces were taken through the central area of each skin test site and embedded in paraffin. Sections six microns in thickness were stained by the Giemsa method.

### F. SEROLOGIC DETERMINATIONS

The serum was obtained from blood drawn just before sacrifice. These sera were tested by the agar-gel precipitin-inhibition technique.<sup>11</sup>

### G. ANAPHYLACTIC TESTING

To determine whether the animals sensitized with dead cells had developed enough circulating antibodies to produce an anaphylactic reaction, the remaining experimental animal from each of the skin testing periods was injected intracardially with one ml of potent coccidioidin. Animals were observed continuously for one hour after intracardiac injection and again at 24, 48, and 72 hours.



## H. PASSIVE TRANSFER

To obtain leukocytes for passive transfer, experimental animals were used 3½ weeks after sensitization with killed arthrospores. Mononuclear cells were obtained from the peritoneal cavity 65 hours after an intraperitoneal injection of 20 ml of sterile nutrient broth.<sup>12</sup> All donors were coccidioidin-positive. Cells of an individual donor animal were given immediately to a recipient animal. Differential cell counts were performed on the peritoneal exudates. Sera from the skin-test-positive animals were used for passive transfer at this time period.

The recipient animals had not been sensitized by either a previous coccidioidin skin test or injection of dead cells. Two animals were used for each serum or cell transfer from a specific donor. One animal received six injections of 0.1 ml each of cells or serum given subcutaneously in the skin of the abdomen. These animals were skin-tested at the site of the cell or serum transfer as previously described beginning 24 hours after the passive transfer. Therefore, each animal had separate skin test reactions of ½, 3, 5, 8, 24, and 48 hours. The second animal receiving only one injection of the passively transferred material was not skin-tested, but the injection site was observed for any reaction. Gross readings were made of the skin test reactions. Animals were sacrificed and tissues were processed as previously described for histopathologic examination.

## III. RESULTS

### A. GROSS OBSERVATIONS

#### 1. 1 Week

One week after sensitization skin tests were negative in all animals.

#### 2. 1½ Weeks

One and one-half weeks after sensitization, one experimental animal developed positive indurations with erythema at 8 and 24 hours. Erythema was a usual but not constant phenomenon in association with induration in animals tested during the following weeks. Erythema developed within 3 hours and progressed in intensity to 24 hours after skin testing in positive reactors.

The other two experimental animals that were negative at this time produced edematous reactions at  $\frac{1}{2}$  to  $\frac{1}{2}$  hour that were 10 x 10 mm. These edematous reactions occurred throughout the experiment in a random manner in approximately half of the experimental and control animals.

The control animal at this  $1\frac{1}{2}$ -week testing period also developed a positive indurated reaction at 8 hours.

### 3. 2 Weeks

Two weeks after sensitization, experimental animals began developing positive reactions beginning at 3 hours and extending to 24 hours. In these animals, as well as in the majority of others tested during the experiment, the skin test reaction at 3 and 5 hours consisted of induration. There was a minority of animals in which the reaction was more edematous at these hours. The 8-hour reactions at this 2-week testing period were the largest size when compared with the other reactions at this 2-week period. After this 2-week period the size of the gross reactions in experimental animals at 3, 5, and 8 hours were consistently greater than those reactions seen at 24 and 48 hours. No consistent pattern in size development between 3 and 5 hours was observed. In the unimmunized control animal of the 2-week testing period the gross reaction to coccidioidin was negative.

### 4. $2\frac{1}{2}$ Weeks

At the  $2\frac{1}{2}$ -week testing period experimental animals continued to develop positive indurations at 3, 5, 8, and 24 hours. The control animal developed edematous reactions at 3 and 5 hours.

### 5. 3 Weeks

The first instance of persistence of the skin test reaction to 48 hours occurred at the 3-week testing period. For some unexplained reason, the control animal also developed positive indurations at all time periods at this 3-week testing.

### 6. $3\frac{1}{2}$ Weeks

Positive reactions continued to be produced at all time periods, except  $\frac{1}{2}$  hour, in experimental animals at  $3\frac{1}{2}$  weeks after sensitization. The control animal at this testing period was completely negative.

## B. HISTOLOGIC REACTIONS

The histologic features of a typical delayed hypersensitivity reaction first appeared at 1½ weeks after immunization and persisted in the sensitized guinea pigs throughout the experiment.

There were two cellular components of the cutaneous reaction to coccidioidin in these sensitized guinea pigs. The first consisted of early neutrophilic infiltration of the dermis and subcutaneous tissues that was uniformly present at 3 hours and usually less marked by 48 hours. The second cellular feature of this reaction was a later and variable mononuclear cell infiltration consisting of lymphocytes, monocytes, and a few plasma cells. This chronic inflammatory cellular reaction appeared later than the neutrophilic reaction and was usually most marked at 24 and 48 hours.

Both the unsensitized control animals and the sensitized test animals manifested the acute inflammatory cell reactions to the injected coccidioidin. The difference between the two was one of degree of mononuclear cell infiltration that occurred sooner and was more marked in the sensitized animals than in control guinea pigs.

The histologic response of the control animals that developed positive gross reactions to skin testing 3 weeks after sensitization was similar to the histologic response observed in other control animals.

## C. SEROLOGIC REACTIONS

The agar-gel precipitin-inhibition reactions were negative with all serum samples from both control and experimental animals.

## D. ANAPHYLACTIC REACTIONS

After intracardiac injection of one ml of coccidioidin, none of the experimental animals exhibited symptoms of anaphylactic shock within one hour. Only one animal, 3½ weeks after sensitization, died within an 18-hour period after the intracardiac injection. This animal showed no symptoms of shock within the first hour after testing.

## E. PASSIVE TRANSFER REACTIONS

Smears made of the peritoneal cellular exudate used to transfer the hypersensitivity skin reactions revealed a predominance of large mononuclear cells. Neither of the two animals that received peritoneal exudate cells alone developed a reaction to them. Animals receiving transferred cells developed gross skin test reactions similar to those

in dead-arthrospore-sensitized guinea pigs. Indurations developed at 3, 5, 8, 24, and 48 hours. Except for one animal at 48 hours, all indurations observed were positive and histologic response in these skin-test areas was similar to that observed in other animals with positive responses. In the animals receiving sera and later skin-tested, no gross delayed reactions were observed except in one animal at 3 hours. The histologic appearance of these negative skin test areas was similar to those of other negative responses.

#### IV. DISCUSSION

Based on the criteria employed in this study, the delayed hypersensitive reaction appears to be a response in the guinea pig to subcutaneous injection of killed arthrospores of *C. immitis*. A working definition that delayed hypersensitivity is an immunologically specific inflammatory reaction that takes some hours to reach a maximum<sup>13</sup> was used. In this investigation, such an inflammatory reaction was produced in the animals sensitized by the injection of killed arthrospores. However, this development was not wholly specific because one of the nonsensitized control animals also produced a similar response. Edema, characteristic of an Arthus reaction, was produced at 30 minutes, but this reaction occurred consistently in both experimental and control animals.

The delayed response can be further characterized histologically. Though there has been some controversy<sup>10</sup> concerning the histologic features of this reaction, it is agreed that the mononuclear cell infiltrate is predominant at the peak of the reaction. In the present investigation the predominant cell type at 24 and 48 hours in the positive skin test areas of sensitized animals was mononuclear. However, this mononuclear reaction was not limited to the sensitized animals only. The nonsensitized control animals also developed a mononuclear response, but to a lesser degree than the experimental animals. The early phase polymorphonuclear response as reported by Boughton and Spector<sup>5</sup> was observed, but a peak at 3 hours was observed in neither the experimental or control animals.

Delayed hypersensitivity also occurs in the absence of demonstrable antibody of the conventional type.<sup>12</sup> In this experiment no antibody was detected by a technique especially devised to detect very small quantities of antibody.<sup>11</sup> The in vivo method, in which coccidioidin was introduced intravascularly, also demonstrated the absence of antibody in the animal that, if present, would cause an anaphylactic reaction. One animal died within 18 hours after intracardiac injection, but since this animal was not observed at the time of death nor necropsied immediately after, the

characteristics of the late febrile response in animals with delayed hypersensitivity to intravenous challenge<sup>14</sup> were not observed. The anaphylaxis test for the presence of circulating antibody has been called a rigid one.<sup>15</sup> If it is negative, as in the present experiment, the reaction is considered to be of the delayed type. Unfortunately in the present investigation it was not possible to demonstrate a late febrile response, which is associated with delayed hypersensitivity.

Since Chase<sup>16</sup> demonstrated the ability of cells in exudates recovered from sensitized animals to transfer delayed hypersensitivity, this method has also been used to characterize this reaction. In this investigation, such a transfer was successfully completed. The gross and histologic reactions as a result of skin testing in the recipient animals were the same as those of the sensitive donor animals. The sensitivity to coccidioidin was not transferred by serum.

It is not known whether improved vaccines against coccidioidomycosis and their subsequent use in humans will elicit a delayed response to skin testing with coccidioidin. Based upon the results obtained in the present investigation, it is suggested that skin testing after immunization might detect positive delayed hypersensitivity in humans and be valuable in determining the effectiveness of the immunization.

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13. ABSTRACT Guinea pigs injected subcutaneously with dead <u>C. immitis</u> arthrospores developed sensitivity to coccidioidin skin testing after 1½ weeks. At that time indurations 5 mm or greater in diameter were seen at 8 and 24 hours. Later indurations, most often with erythema, were observed at 3, 5, 8, 24, and 48 hours. Gross reactions were not completely specific to the immunized animals and one nonimmunized control animal developed similar indurations. Histologic features of a delayed hypersensitivity also appeared 1½ weeks after immunization. This reaction consisted first of early neutrophilic infiltration of the dermis and subcutaneous tissues uniformly present at 3 hours and less marked by 48 hours. The second cellular feature was a later, variable mononuclear cell infiltration and was usually most marked at 24 and 48 hours. The acute inflammatory reaction was produced in both sensitized and control animals. The two groups differed in the degree of mononuclear cell infiltration, which occurred sooner and was more marked in the sensitized animals than in the controls.  All agar-gel precipitin-inhibition reactions were negative on sera from all animals tested.  No immediate anaphylactic reaction was produced in immunized animals by intravascular injection of coccidioidin.  Peritoneal cellular exudate, predominantly large mononuclear cells, from immunized, coccidioidin-positive donors transferred the hypersensitivity to control animals. Sera from similar immunized coccidioidin donors failed to transfer the hypersensitivity.		

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